Markers of endothelial injury and platelet microparticles are distinct in patients with stable native coronary artery disease and with cardiac allograft vasculopathy

Neha Singh[1], Johan Vanhaecke[2], Johan Van Cleemput[3], Bart De Geest[4]

[1]: Centre for Molecular and Vascular Biology, Department of Cardiovascular Sciences, Catholic University of Leuven, Leuven, Belgium.
This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.
[2]: Cardiology, Department of Cardiovascular Sciences, Catholic University of Leuven, Leuven, Belgium.
This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.
[3]: Cardiology, Department of Cardiovascular Sciences, Catholic University of Leuven, Leuven, Belgium.
This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.
[4]: Centre for Molecular and Vascular Biology, Department of Cardiovascular Sciences, Catholic University of Leuven, Leuven, Belgium.
This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

Address for correspondence: Bart De Geest MD, PhD
Centre for Molecular and Vascular Biology
Department of Cardiovascular Sciences
Catholic University of Leuven
Campus Gasthuisberg
Herestraat 49 bus 911
3000 Leuven
Belgium
Tel.: 00 32 16 372059
Fax.: 00 32 16 345990
E-mail: bart.degeest@med.kuleuven.be

Word count: 999

Grant support: This work was supported by grant G.0529.10N of the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen.

Conflict of interest: none

Key words: coronary artery disease, cardiac allograft vasculopathy, endothelial injury, endothelial activation, endothelial microparticles, platelet microparticles

Endothelial injury is assumed to play a key role in the initiation and progression of both native coronary artery disease (CAD) and cardiac allograft vasculopathy (CAV) [1, 2]. Biochemical surrogate biomarkers of endothelial injury (e.g. von Willebrand factor, soluble thrombomodulin, soluble vascular cell adhesion molecule, soluble intercellular adhesion molecule, and soluble E-selectin) do not discriminate between endothelial activation and irreversible endothelial damage and lack endothelial specificity [3]. In contrast, cellular biomarkers of endothelial injury (circulating endothelial microparticles (CEMPs) and circulating endothelial cells (CECs)) are endothelial-specific. Microparticles are 0.1 µm to 1 µm large membrane vesicles that are released following cell activation or apoptosis [4]. In contrast to CEMPs, CECs constitute a parameter of irreversible damage of the endothelium [5, 6, 7]. CECs are mature endothelial cells that originate by detachment from the endothelial monolayer as a result of an endothelial insult. Thus, by combined quantification of CEMPs and CECs, it is possible to evaluate to which extent endothelial injury represents endothelial activation or endothelial denudation. We investigated whether a distinct pattern of endothelial injury (endothelial activation versus endothelial denudation) is observed in native CAD and CAV.

Eighty patients with clinically stable native coronary artery disease (CAD) and thirty heart transplant recipients with cardiac allograft vasculopathy (CAV) were recruited in the current cross-sectional study. Stable native CAD patients were defined by the presence of at least one stenosis of 50% or more demonstrated by diagnostic coronary angiography. CAV was diagnosed in heart transplant recipients undergoing coronary angiography between 5 and 15 years after heart transplantation [8]. CAV was graded according to the ISHLT working formulation of a standardized nomenclature for CAV-2010 [9]. The study protocol conforms to the ethical guidelines of the 1975

Declaration of Helsinki as reflected by the *a priori* approval of the protocol by the Ethics Committee of the University Hospital Gasthuisberg. Written informed consent was obtained from all participants. The reference control group included 25 healthy control subjects (12 males and 13 females). Average age of healthy controls was 43.2 ± 2.0 years.

Clinical characteristics, laboratory parameters, and medical therapy in patients with stable native CAD and heart transplant recipients with CAV are summarized in Table 1. Patients with native CAD were 4.7 years ($p<0.05$) older, had a lower prevalence of hypertension ($p<0.0001$), and a higher body mass index than patients with CAV ($p<0.05$). C-reactive protein levels were 6.32-fold ($p<0.001$) higher in heart transplant recipients with CAV than in patients with native CAD. Lipoprotein levels were very similar and statin use was generalized in both conditions. The use of antiplatelet drugs was generalized in patients with native CAD and was restricted to one heart transplant recipient with CAV.

The number of circulating endothelial cells, endothelial microparticles, and platelet microparticles was quantified by flow cytometry using a BD FACSCantoII flow cytometer and BD FACSDIVA software version 1.2.6 (BD Biosciences, San Jose, California, USA). The geometric mean of the concentration of circulating endothelial ($CD45^{-}$ $CD31^{bright}$ $VEGFR\text{-}2^{+}$) cells (CECs) was 2.90-fold ($p<0.001$) and 2.34-fold ($p<0.05$) higher in patients with native CAD and with CAV, respectively, compared to healthy controls (data not shown). No significant difference of Annexin V negative CECs and of Annexin V positive (apoptotic) CECs was observed between patients with native CAD and transplant recipients with CAV. Taken together, the number of CECs as a parameter of irreversible endothelial damage is similarly increased in both types of arteriosclerosis.

Circulating endothelial ($CD42a^-$ $CD144^+$) microparticles (CEMPs), Annexin V negative CEMPs, and Annexin V positive CEMPs were elevated in patients with native CAD and transplant recipients with CAV compared to healthy controls as illustrated in Figure 1A, Figure 1B, and Figure 1C, respectively. The concentration of CEMPs (Figure 1A) and Annexin V negative CEMPs (Figure 1B) was 45.8% ($p<0.01$) and 59.2% ($p<0.01$) higher, respectively, in transplant recipients with CAV than in patients with native CAD. No significant difference in Annexin V positive CEMPs was observed between patients with native CAD and CAV (Figure 1C). Taken together, the selective increase of Annexin V negative CEMPs in transplant recipients with CAV compared to patients with native CAD is compatible with more pronounced endothelial cell activation in the former.

Platelets can adhere to dysfunctional endothelium, exposed collagen, and macrophages, and promote initiation and progression of atherosclerosis [1, 10]. Platelet microparticles are a marker of platelet activation. We compared circulating platelet ($CD61^+$) microparticles (CPMPs) in patients with native CAD and with CAV. Circulating platelet microparticles (CPMPs), Annexin V negative CPMPs, and Annexin V positive CPMPs were significantly lower in patients with native CAD compared to healthy controls and transplant recipients with CAV as illustrated in Figure 1D, Figure 1E, and Figure 1F, respectively. The median value of total CPMPs in patients with native CAD was 69.4% ($p<0.001$) and 71.6% ($p<0.001$) lower compared to healthy controls and transplant recipients with CAV, respectively. This decline was observed both for Annexin V negative CPMPs (Figure 1E) and Annexin V positive CPMPs (Figure 1F). No difference in any of these parameters was

observed between healthy controls and transplant recipients with CAV. Similar results were obtained when $CD42a^+$ $CD31^+$ CPMPs were quantified (data not shown).

Since all patients with native CAD and only one patient with CAV were taking antiplatelet drugs, this suggested that the observed difference in CPMPs is due to this class of medication. The concentration of $CD61^+$ CPMPs (60.8/µl) in the CAV patient taking acetylsalicylic acid was in the order of magnitude of patients with native CAD. To further confirm the effect of acetylsalicylic acid on CPMPs, an additional quantification was performed in 11 heart transplant recipients taking this drug. The median value of $CD61^+$ CPMPs (53.0/µl) in these heart transplant recipients was similar compared to patients with native CAD.

In conclusion, the selective increase of Annexin V negative CEMPs and the absence of a difference in Annexin V positive CECs strongly suggest increased endothelial activation but not endothelial apoptosis in CAV positive patients compared to stable CAD patients. Use of antiplatelet drugs likely underlies the strikingly lower levels of CPMPs in patients with native CAD.

ACKNOWLEDGMENTS

This work was supported by grant G.0529.10N of the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen. The funding source had no role in the collection, analysis and interpretation of data**.**

REFERENCES

[1] Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999;340:115-26.

[2] Schmauss D, Weis M. Cardiac allograft vasculopathy: recent developments. Circulation. 2008;117:2131-41.

[3] Burger D, Touyz RM. Cellular biomarkers of endothelial health: microparticles, endothelial progenitor cells, and circulating endothelial cells. J Am Soc Hypertens. 2012;6:85-99.

[4] Mause SF, Weber C. Microparticles: protagonists of a novel communication network for intercellular information exchange. Circ Res. 2010;107:1047-57.

[5] Boos CJ, Lip GY, Blann AD. Circulating endothelial cells in cardiovascular disease. J Am Coll Cardiol. 2006;48:1538-47.

[6] Blann AD, Woywodt A, Bertolini F, et al. Circulating endothelial cells. Biomarker of vascular disease. Thromb Haemost. 2005;93:228-35.

[7] Rogers NJ, Lechler RI. Allorecognition. Am J Transplant. 2001;1:97-102.

[8] Singh N, Van Craeyveld E, Tjwa M, et al. Circulating apoptotic endothelial cells and apoptotic endothelial microparticles independently predict the presence of cardiac allograft vasculopathy. J Am Coll Cardiol. 2012;60:324-31.

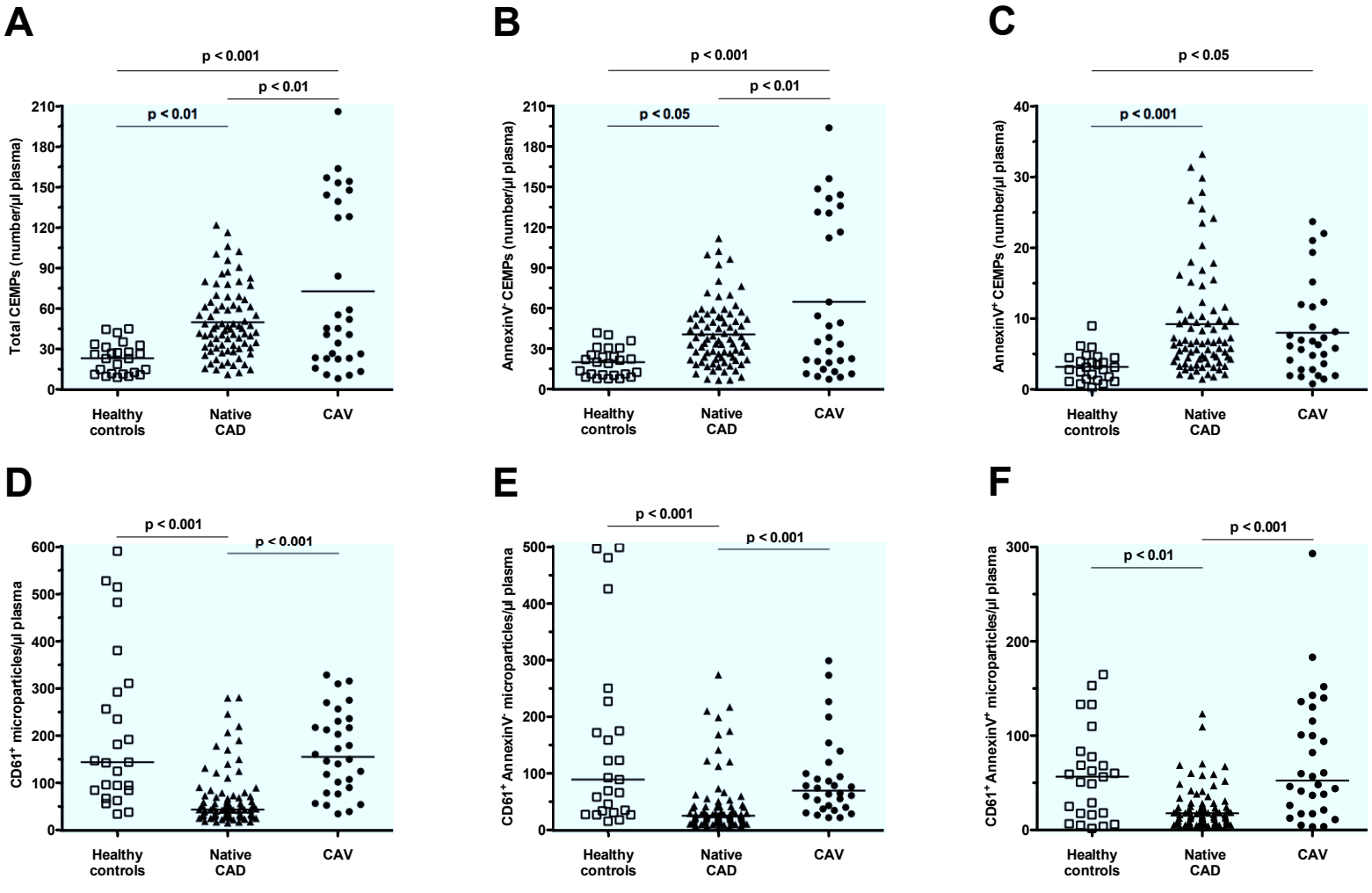
[9] Mehra MR, Crespo-Leiro MG, Dipchand A, et al. International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for cardiac allograft vasculopathy-2010. J Heart Lung Transplant. 2010;29:717-27.

[10] Ross R, Glomset JA. The pathogenesis of atherosclerosis (first of two parts). N Engl J Med. 1976;295:369-77.

LEGEND TO THE FIGURE

Figure 1. Individual value bar graphs illustrating a comparison between healthy controls (n=25), stable native CAD patients (n=80), and heart transplant recipients with CAV (n=30) of circulating endothelial microparticles (CEMPs) and of CD61$^+$ circulating platelet microparticles (CPMPs) Total CEMPs, Annexin V$^-$ CEMPs, and Annexin V$^+$ CEMPs are illustrated in panel A, B, and C, respectively. Horizontal lines in these three upper panels indicate the means. Total CD61$^+$ CPMPs, CD61$^+$ Annexin V$^-$ CPMPs, and CD61$^+$ Annexin V$^+$ CPMPs are shown in panel D, E, and F, respectively. Horizontal lines in these three lower panels indicate the medians.

Figure(s)


A
p < 0.001
p < 0.01
p < 0.01
Total CEMPs (number/µl plasma)
210
180
150
120
90
60
30
0
Healthy controls
Native CAD
CAV
B
p < 0.001
p < 0.01
p < 0.05
AnnexinV⁻ CEMPs (number/µl plasma)
210
180
150
120
90
60
30
0
Healthy controls
Native CAD
CAV
C
p < 0.05
p < 0.001
AnnexinV⁺ CEMPs (number/µl plasma)
40
30
20
10
0
Healthy controls
Native CAD
CAV
D
p < 0.001
p < 0.001
CD61⁺ microparticles/µl plasma
600
500
400
300
200
100
0
Healthy controls
Native CAD
CAV
E
p < 0.001
p < 0.001
CD61⁺ AnnexinV⁻ microparticles/µl plasma
500
400
300
200
100
0
Healthy controls
Native CAD
CAV
F
p < 0.001
p < 0.01
CD61⁺ AnnexinV⁺ microparticles/µl plasma
300
200
100
0
Healthy controls
Native CAD
CAV

Table(s)

Table 1. Clinical characteristics, laboratory parameters, and hypolipidemic and antiplatelet therapy in patients with stable native CAD and in transplant recipients with CAV.

	Patients with stable native CAD (n=80)	**Patients with CAV (n=30)**	**P value**
Age at inclusion in the study (years)	69.6 ± 1.2	64.9 ± 2.1	0.0459
Sex (male/female)	63 (78.8%)/17 (21.3%)	24 (80.0%)/6 (20.0%)	1.00
Current smoker (%)	7.5%	0%	0.186
Hypertension (%)	47.5%	100%	<0.0001
Diabetes (%)	25.0%	36.7%	0.242
Body mass index (kg/m^2)	27.5 ± 0.6	25.5 ± 0.5	0.0337
Platelet count ($10^9/L$)	222 ± 5	217 ± 12	0.150
Leukocyte count ($10^9/L$)	7.19 ± 0.22	6.87 ± 0.24	0.398
Monocyte count ($10^9/L$)	0.435 ± 0.029	0.313 ± 0.039	0.012
Lymphocyte count ($10^9/L$)	1.94 ± 0.08	1.28 ± 0.12	<0.0001
Neutrophil count ($10^9/L$)	4.22 ± 0.20	3.78 ± 0.36	0.213
Creatinine (mg/dl)	1.05 ± 0.03	1.60 ± 0.08	<0.0001
CRP (mg/l)	1.95 ± 0.18	12.4 ± 3.3	<0.0001
Cholesterol (mg/dl)	160 ± 4	156 ± 6	0.548
Triglycerides (mg/dl)	132 ± 7	125 ± 12	0.557
HDL cholesterol (mg/dl)	50.2 ± 1.8	54.2 ± 3.4	0.353

LDL cholesterol (mg/dl)	83.6 ± 3.0	77.1 ± 4.0	0.402
Acetylsalicylic acid (%)	88.8%	3.33%	<0.0001
Statins (%)	95.0%	100%	0.573
Clopidogrel (%)	13.8%	0%	0.0334

Data are expressed as means ± SEM for continuous variables.